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PRINCIPAL INVESTIGATOR: Harvey L. Bumpers, MD

CONTRACTING ORGANIZATION: Morehouse School of Medicine
Atlanta, GA 30310

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HARVEY L. BUMPERS, MD

Email: hbumpers@msm.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
MOREHOUSE SCHOOL OF MEDICINE
720 WESTVIEW DR, SW
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14. ABSTRACT
Introduction. We have shown that a novel peptide from HIV-1 Nef (NefM1) induces apoptosis through the CXCR4 receptor. We would like to exploit this as a cancer therapeutic agent. Methods. Breast cancer cell lines, MDA-MB231 (231), MDAMB468 (468), MCF-7, DU4475, and HMEC were each evaluated for their response to NefM1. Apoptosis was assessed using TUNEL staining and caspase-3 activation. The presence of CXCR4 receptors on the tumor cells was determined using immunohistochemistry and PCR analyses. Xenografts derived from CXCR4+ cells were propagated in SCID mice and evaluated for the persistence of the receptor and the effects of NefM1 on growth and metastasis. The growing tumors underwent volumetric measurements weekly. and comparisons were made between those treated with NefM1 biweekly, by i.p. injections, and those untreated. Results. Breast cell lines that were positive for CXCR4 receptors all underwent apoptosis when treated with Nef, as did those CXCR4-negative cells that were transfected with CXCR4. Breast xenografts derived from 231 cells demonstrated smaller primaries and significantly smaller metastatic tumors in the Nef treated group. Conclusion. NefM1 causes apoptotic reduction in in vitro and in vivo growth of breast cancer cells and tumor xenografts, respectively.

15. SUBJECT TERMS
CXCR4, BREAST CANCER, NEFM1, NEF PEPTIDE

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Introduction

Over the past thirty years there has been a steady increase in the incidence of breast cancer. It is the second most frequent cause of cancer-related death in American women. There is decreased survival with increasing stage of disease and stage IV, distant metastasis, is incurable. There is specific evidence linking the CXCR4 receptor to microvascular endothelial proliferation in human breast cancer. The chemokine receptor/chemokine ligand, CXCR4/SDF-1a, pair has also been shown to be critically involved in a growth factor-regulated signaling system in endothelial cells that mediates important steps in vascular remodeling[1]. Vascular remodeling and angiogenesis is a critical step in the establishment and subsequent viability of tumors[1]. Furthermore, the expression of CXCR4 on the cell surface appeared to promote metastasis by acting directly on migration and invasion of these tumor cells.

We have identified a Nef protein that consist of a 10 amino acid peptide that induces apoptosis in cancer cells [2,3]. The apoptotic activity of Nef M1 peptide is mediated through the CXCR4 receptor, and Nef can effectively compete with CXCR4's natural ligand, SDF-1α[4], and induce apoptosis. Muller et al[4], found that CXCR4 was highly expressed in human breast carcinoma cells whereas the normal primary mammary epithelial cells lack expression of the CXCR4 receptor. The NefM1 peptide has been shown to be highly cytotoxic to a number of different human cancer cell lines *in vitro*, including breast cancer cells lines such as MDA-MB-231, MCF-7, and MDA-MB-468 transfected with the CXCR4 gene. Human breast cancers can be maintained as xenografts in the SCID mouse for long periods of time. These tumors can be generated subcutaneously, but metastasizes intraperitoneally and to the liver when implanted in the gonad fat pad of the SCID mouse. This breast model is used to determine the effect of this novel peptide on primary tumor growth, and metastasis. We hypothesize that the active Nef-1 peptide will slow the growth of primary breast cancer through apoptosis as well as by blocking the angiogenic ability of the tumor, and it will cause inhibition of tumor metastasis.

Body of Report

The research project established three specific aims:

AIM I: To determine the NefM1's ability to slow/inhibit primary and metastatic tumors derived from established cell lines. A SCID mouse model using established breast cancer cells injected sub Q, intrasplenic, and via the gonad fat will be used to evaluate the metastatic process. The ability of the tumors to metastasize from the flank to the chest wall or from the spleen or gonad fat to the liver in the presence of systemic NefM1 will be assessed. Tissues from these tumors will be analyzed regarding the quantity of CXCR4 present and the occurrence of apoptosis after exposure to NefM1.

AIM II: To determine the NefM1's ability to slow/inhibit primary and metastatic growth of tumors derived from fresh surgical specimens. Surgical specimens of human breast cancer will be implanted in the subcutaneous tissue and intraperitoneally using the gonad fat pad of virgin female mice. The human cancer specimen implants will be analyzed for CXCR4. The growth patterns of the primary xenograft and metastatic lesions will be assessed first. The use of NefM1 peptide on the growing tumors will then be carried out to determine its ability inhibit or alter the growth processes.

AIM III: To characterize the NefM1's effect on vascular density of tumors from established breast cancer cell lines. Primary breast xenografts generated from cell lines and surgical specimens and treated with Nef in vivo will be evaluated for vascular density. Tumors will be evaluated during the early (2-3 days after inoculation) and late (30 days after inoculation or when large tumors develop) phases of growth. Staining techniques that utilizes light microscopy and immunohistochemistry will be used to quantitate the level of angiogenesis.

The initial work concentrated on fulfilling specific aim I by characterizing a diverse population of normal and malignant breast cells that would be used to generate tumors subcutaneously and intraperitoneally. Five different cell lines MDA-MB-231 (CXCR4 +) cells, MDA-MB-468 (CXCR4 -) cells, HMEC–primary human mammary epithelium cells (CXCR4 -), MCF 7 – human mammary carcinoma (CXCR4 +), and DU 4475 – human mammary carcinoma (CXCR4 +) were cultured. The following studies were done during this phase of the project. (*i*) The cell lines were evaluated for confirmation of their CXCR4 status using anti-CXCR4 monoclonal antibodies and by RT-PCR analysis of CXCR4 mRNA expression. *(ii)* The ability of the Nef M1 to induce apoptosis in these cells was studied using Tunel assay and western blot analysis of caspase-3 activation. *(iii)* A dose response was generated to assess the Nef M1 peptide's cytotoxicity on these cells. *(iv)* The cell lines were injected subcutaneously and intraperitoneally (spleen and gonad fat) to generate primary tumors and metastasis, respectively. All work was done in the

laboratory of the Principle Investigator, Dr. Bumpers. The Co-Investigators, Drs. Bond and Huang, participated in the project as noted in the grant proposal.

Results

Nef M1 dose response analysis. Dose response to full Nef protein and the Nef M1 peptide to a concentrations 0 ng, 0.01ng, 0.1 ng, 1ng, 10 ng and 100 ng/ml in CXCR4 negative (MDA-MB-468, HMEC) and positive (MDA-MB-231, MCF7) breast cancer cell lines for 24 hours , and then TUNEL Assay, figure 1. The increased concentration of the whole protein (Nef) and the peptide (NefM1) each directly correlates with apoptosis in MDA-MB-231 and MCF-7 cells.

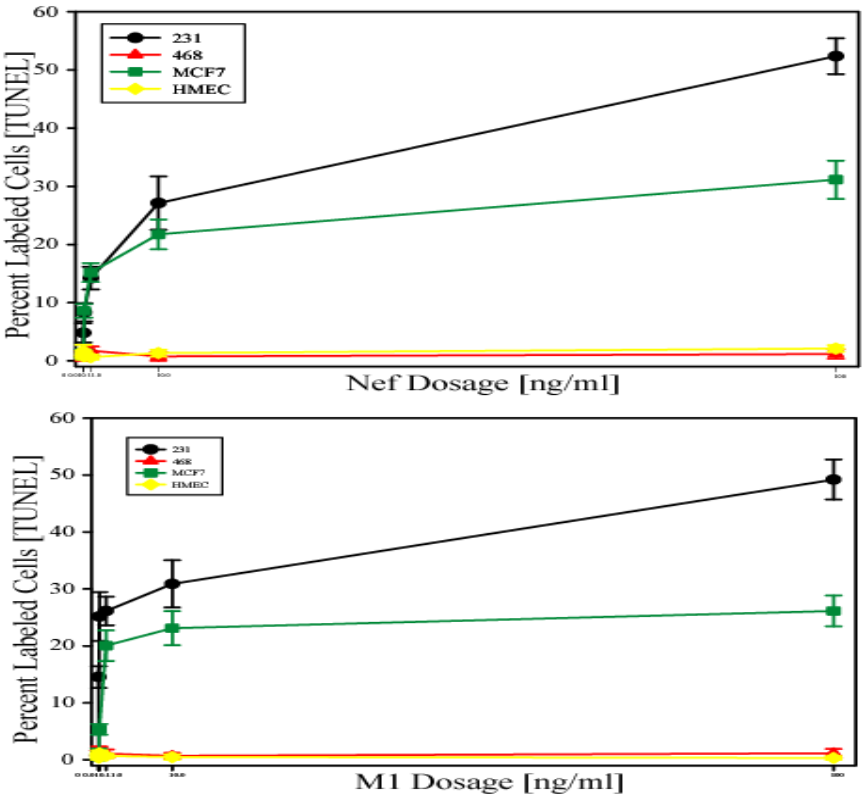
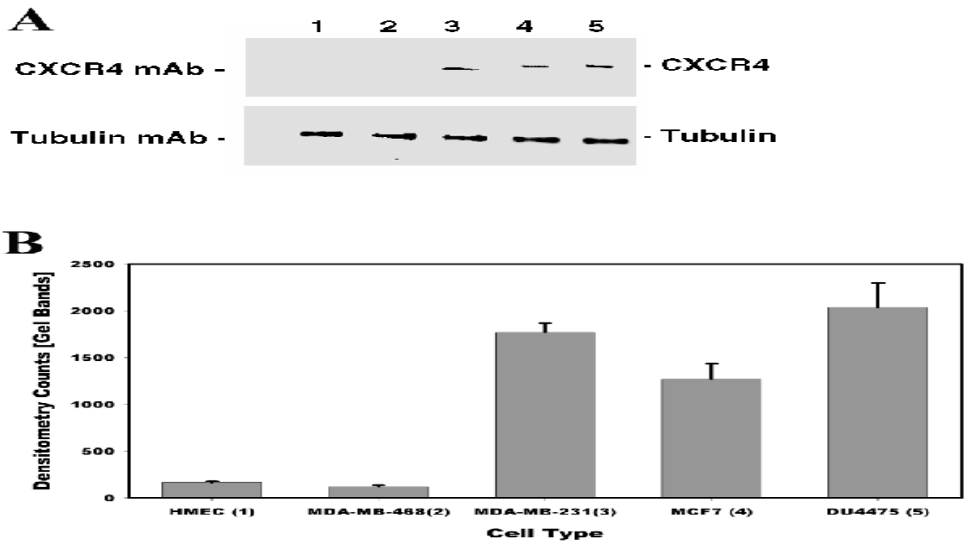


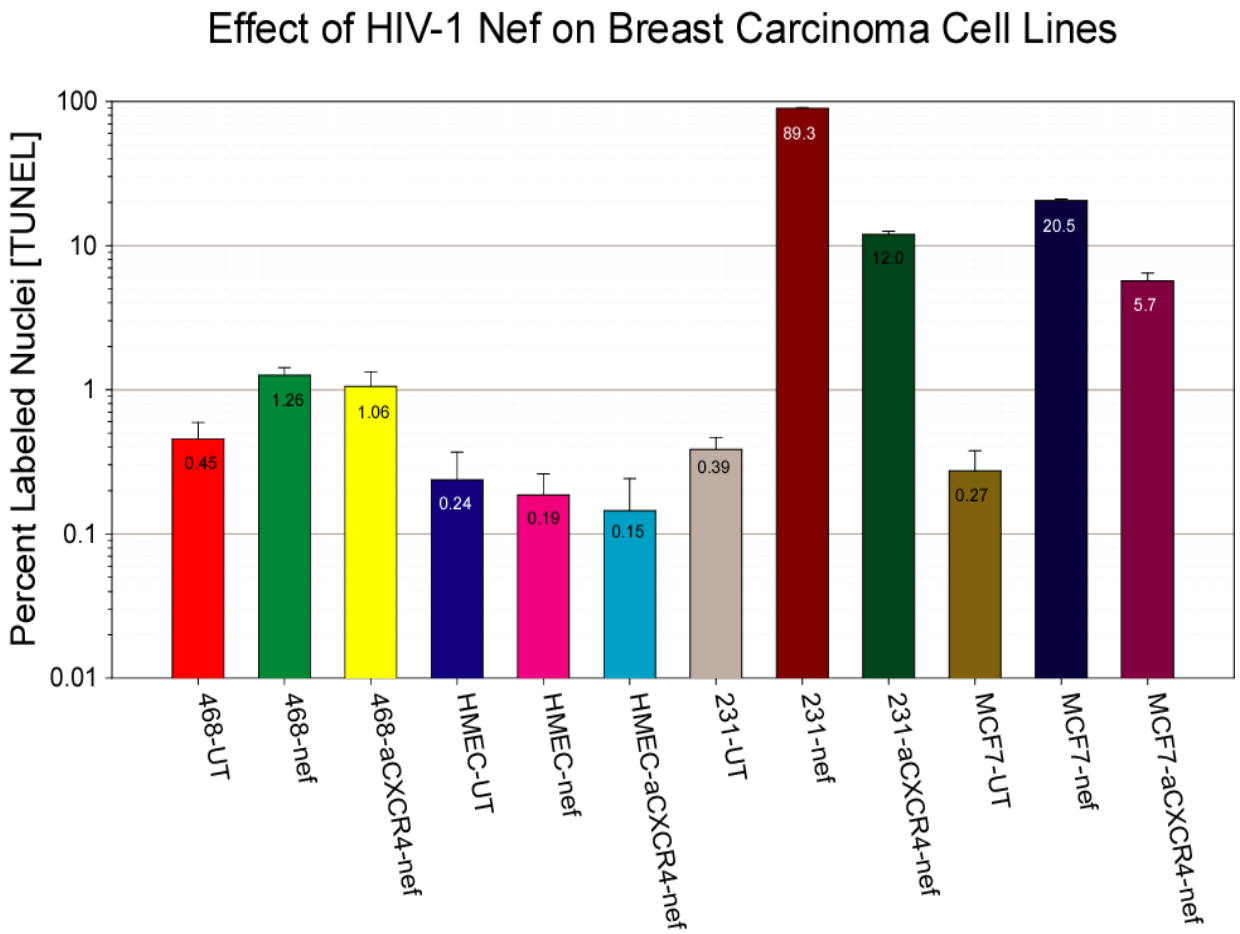
Figure 1. Dose Response to full Nef protein and the Nef M1 peptide in CXCR4 negative (468, HMEC) and positive (231, MCF7) breast cancer cell lines

Western blot analysis further confirms those cell lines containing CXCR4 receptors. The MDA-MB-231, MDA-MB-468, MCF7, DU4475 and HMEC cells were grown to 80%

confluence in 35mm plates for 2 days. Cells were collected and centrifuged for 500 xg for 5 minutes at 4^{o}C. The pellet was added 200 ul of lysis solution. Twenty five microliters of each sample was separated by SDS-PAGE on a 4-20% Tris-HCl Criterion precast gel, and electrophoretically transferred to a nitrocellulose membrane and probed with a primary mouse anti-human CXCR4 antibody and followed with a secondary anti-mouse IgG (H+L) conjugated HRP antibody. The blot was stripped and hybridized with a monoclonal mouse anti-α-tubulin antibody followed by a HRP-conjugated goat anti-mouse IgG (H + L) by Western blot analysis. Lane 1. HMEC, lane 2. MDA-MB-468, lane 3. MDA-MB-231, lane 4. MCF7 and lane 5. DU4475. (B). Densitometric Analysis of CXCR4 Bands. Images were scanned into Adobe Photoshop 5.0.2, and densitometry was performed using Scion Imaging software (Scion Corporation, Frederick, MD.) and normalized against intracellular tubulin. Therefore the breast cancer cell lines that we are working with that should be susceptible to cytotoxicity from the Nef M1 peptide are MDA-MB-231, MCF-7, and DU4475.



In figure 3 below the treatment effect of HIV-1 Nef M1 on breast carcinoma cell lines by TUNEL Assay is illustrated. The untreated CXCR4 positive cells responded similarly to the CXCR4 negative cell. A treatment difference was seen only in those CXCR4 receptor positive cells. Though not shown in this figure, when MDA-MB-468 cells were transfected with the CXCR4 gene apoptosis then occurred secondary to exposure to the Nef M1 peptide. Briefly, the methodology was as follows, the MDA-MB-468, HMEC, MDA-MB-231 and MCF7 cells were grown to 80% confluence in 35mm plates for 24 hours. Cells were treated with either HIV-1 Nef protein (50ng/ml) at 37^{o}C for 24 hours or pre-treated with an anti-CXCR4 monoclonal antibody (1:1000) for 30 minutes, and washed by PBS, and then treated with HIV-1 Nef protein at 37^{o}C for 24 hours, and followed by TUNEL assay.



To simplify Identification of CXCR4 receptors on breast cancer cells immunohistochemistry was used. Using IHC we could demonstrate that MB-231 cells were strongly positive for CXCR4 receptors. No CXCR4 is expressed on MB-468 cells. The presence of CXCR4 is expressed as a brightly fluorescent green stain as shown figure 4. Breast cancer xenografts derived from these cells likewise had strong expression of CXCR4 positivity. See RT-PCR analysis, figure 5.

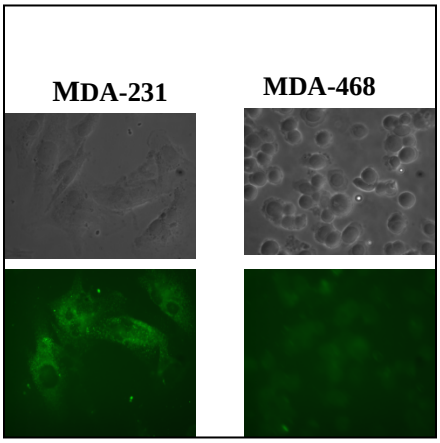


Figure 4

In order to confirm definitively that apoptosis in response to Nef M1 was responsible for cell death in the CXCR4 positive cells (MDA-MB-231, MCF-7) caspase-3 activation was evaluated. The results of analysis are noted in figure 5. This shows the effect of Nef peptides on MDA-MB-231 cells by caspase-3 activation analysis. We did a Western blot analysis for detection of Caspase-3 activation in MDA-MB-231 cells. The difference in this study is that a known CXCR4 positive cell line was used to evaluate our Nef peptide in comparison to other motifs in the same protein. The amino acids accounting for the Nef M1 motif are truly the apoptotic peptide as exemplified by the caspase-3 activation in the cells. The cells were grown to 80% confluence in T-25mm at 37^{o}C for 2-4 days. Cells were treated with (A) 24 hours (B) 48 hours. Twenty micrograms (μg) of each sample total protein was separated by SDS-PAGE on a 4-20% Tris-HCl Criterion precast gel, and electrophoretically transferred to a nitrocellulose membrane by Western blot analysis. Lane 1. Untreated, Lane 2. sM1, Lane 3. Nef 171-180, Lane4. Nef protein, Lane 5. Nef 41-60, Lane 6. Nef M1. Caspase-3 activation was determined by cleavage of 35 kDa pro-Caspase-3 protein, and two smaller 17 kDa and 12 kDa active Caspase-3 protein,

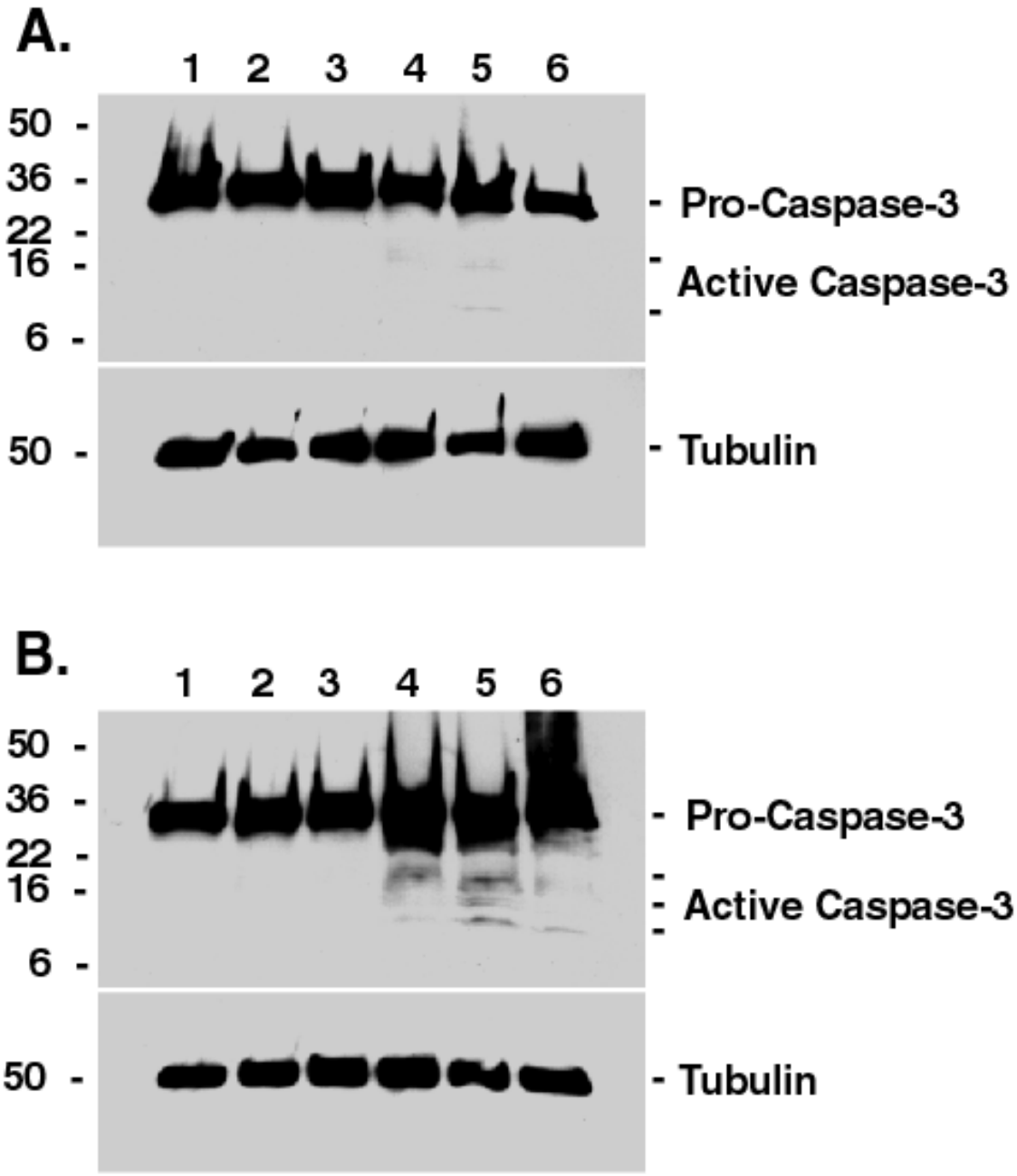
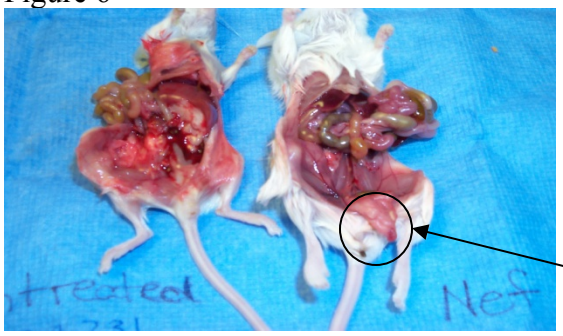


Figure 5.
Effect of Nef peptides on MDA-MB-231 Cells – Caspase 3 Analysis
Each lane contains 20 ug of total protein.
A. 24 hours
B. 48 hours
Lane 1 ut, Lane2. nef s50-60, Lane3. nef 171-180, Lane4. nef protein, Lane5. nef 41- 60, Lane6. nef 50-60

Breast cancer xenografts implanted in the gonad fat pad of mice will grow and metastasize to the liver as demonstrated by the Principal Investigator. The mice below were either untreated (on the left) or treated with NefM1 (on the right) starting at the time of tumor implantation. The untreated mouse developed diffuse intraperitoneal metastasis and in the treated mouse no metastasis occurred and the primary tumor did not propagate. A normal appearing gonad fat pad, which had been implanted with cancer, is hanging from the pelvis next to the left leg (illustrated by a circle). The gonad fat in the untreated mouse is incorporated in the tumor growing in the pelvis.

Figure 6



We have shown Nef M1 peptide to be highly cytotoxic to a number of different human cancer cell lines *in vitro*, including breast cancer cells lines such as MDA-MB-231, MCF-7, Du4475, and MDA-MB-468 transfected with the CXCR4 gene (unpublished evidence). This has been shown to be directly proportional to the concentration of CXCR4 on those cells surfaces (unpublished evidence). We have studied the effects of the peptide on the growth of primary colorectal cancer xenografts generated from surgical specimens taken directly from the operating room and implanted in SCID mice. We have found it to be a potent inhibitor of tumor growth[5,6]. Similar results are expected with breast cancer xenografts, since the breast cancer cellular components studied here undergo apoptosis when exposed to the Nef peptide.

Regarding implantation and propagation of human breast cancer in SCID mice**,** we have demonstrated that surgical specimens of human breast cancer can be implanted and propagated in the SCID mouse using the gonad fat pad[7]. Metastatic disease can also be generated from these xenografts. These breast cancer cells (MDA-MB-231) can also be injected into the spleen to generate primary and metastatic tumors.

There is already a body of literature on the molecules that regulate CXCR4, and the signaling that regulates its expression. However, this project starts with the premise that this receptor is important in cell proliferation, angiogenesis, and metastasis. This study addresses the issue of targeting that receptor for development of cancer therapy.
As we embarked on the first year of this project our initial goal was to show that Nef's activity is mediated through the chemokine co-receptor CXCR4 which is identified for all the breast cancer cells used in our studies. We are satisfied that this has been achieved.

We have most recently developed primary breast cancers from MDA-MB 231 and DU4475 cells as subcutaneous malignancies. Currently two studies are ongoing to evaluate the systemic effects of Nef on these subcutaneous primary tumors and the effect of local treatment, intratumoral injection. Early data acquired regarding intratumor injections indicate an interruption of tumor vascularity. The histopathology is yet to be completed.

Year 2 (2009-2010) of the project will complete the evaluation of the use of Nef with breast cancer cell lines, and we will also begin specific aim II that evaluates these same studies but with human surgical specimens. As previously noted in aim II we will take surgical specimens directly from the operating room and implant them in SCID mice to achieve xenografts. Those tumors will then be studied for their ability to propagate and metastasize, and the inhibitory affects of Nef M1 on both the growth and metastasis.

Key Research Accomplishments

- CXCR4 receptor status was documented or confirmed for five breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7, DU4475, HMEC)
- The ability of Nef M1 to induce apoptosis in breast cancer cells that contains the CXCR4 receptor was demonstrated
- Breast cancer xenografts tissue derived from the cell lines were also shown contained the CXCR4 receptor
- Primary tumors were produced in the subcutaneous tissue of SCID mice from established cell lines (MB-231, MCF-7, DU4475)
- Techniques for establishing intraperitoneal and hepatic metastasis from these breast cancer cell line were established using the mouse gonad fat and intrasplenic inoculations.

These accomplishments can be used to help achieve specific aim II in which we will be studying surgical specimens of human breast cancer. The effect of Nef M1 on the primary tumor growth and metastasis from established cell lines will continue. This will include intratumoral injections of NefM1. Surgical specimens will undergo similar experimentation over the next year of research.

Reportable Outcomes

- Presentation at Surgical Grand Rounds, Morehouse School of Medicine, Atlanta, GA
- Abstract of Preliminary data presented at Society of Black Academic Surgeons, Chicago, 2008. *Exploitation of the CXCR4 Receptor in Breast Cancer*
- Manuscript in preparation for submission this summer
- Abstract submissions planned for Society of Surgical Oncology and American Association for Cancer Research based on data already collected and presented in this report.

Conclusion

CXCR4 receptors on established human breast cancer cell lines can be documented with immunohistochemistry and RT-PCR analysis and these techniques should apply to surgical specimens of human breast cancer. Furthermore, the Nef M1 peptide binding to CXCR4 receptor can be demonstrated and results in a significant inhibitory effect on the growth of breast cancer cells and the tumor tissue counterpart by inducing apoptosis. We have established techniques and protocols for moving on to specific aim II.

"so what" statement

The NefM1 peptide binds to the CXCR4 receptor which is present on many human tumors and through signaling it causes apoptosis within the tumor. This offers the possibility of developing a novel breast cancer therapeutic regimen. Since this peptide can displace the natural ligand and bind to a specific receptor (CXCR4) it may also developed as a therapeutic agent that can be used to target tumor implantation, progression and metastasis.

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